THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 41, pp. 36076–36085, October 14, 2011

Akt Fine-tunes NF-κB-dependent Gene Expression during T Cell Activation*[S]

Received for publication, May 10, 2011, and in revised form, July 28, 2011 Published, JBC Papers in Press, August 23, 2011, DOI 10.1074/jbc.M111.259549

Jing Cheng[‡], Binh Phong[‡], David C. Wilson[§], Raphael Hirsch[§], and Lawrence P. Kane[‡1]

From the [‡]Dept. of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261and the [§]Division of Rheumatology, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania 15224

Background: The precise role of the Akt kinase in NF-κB induction by the TCR and CD28 is still unclear.
Results: We have found that Akt makes a quantitative contribution to NF-κB induction in T cells, selectively impacting a subset of downstream genes.
Conclusion: Although Akt is not a canonical member of the NF-κB pathway, it can modulate NF-κB signaling and transcription.
Significance: These findings may open the way to more selective modulation of NF-κB-dependent pathways.

Activation of the NF-κB signaling pathway is critical for leukocyte activation and development. Although previous studies suggested a role for the Akt kinase in coupling the T cell antigen receptor and CD28 to NF-κB activation in T cells, the nature of the role of Akt in this pathway is still unclear. Using a targeted gene profiling approach, we found that a subset of NF-κB-dependent genes required Akt for optimal up-regulation during T cell activation. The selective effects of Akt were manifest at the level of mRNA transcription and p65/RelA binding to upstream promoters and appear to be due to altered formation of the Carma1-Bcl10 complex. The proinflammatory cytokine TNF-α was found to be particularly sensitive to Akt inhibition or knockdown, including in primary human blood T cells and a murine model of rheumatoid arthritis. Our findings are consistent with a hierarchy in the expression of NF-κB-dependent genes, controlled by the strength and/or duration of NF-κB signaling. More broadly, our results suggest that defining the more graded effects of signaling, such as those demonstrated here for Akt and the NF-κB pathway, is important to understanding how cells can fine-tune signaling responses for optimal sensitivity and specificity.

The proto-oncogenic kinase known as Akt (or protein kinase B) is a serine/threonine kinase activated downstream of the lipid kinase PI3 kinase, which is recruited to ligated growth factor and antigen receptors. Dysregulation of the Akt pathway has been demonstrated in numerous cancers, including some leukemias and lymphomas (1, 2), and many downstream targets for Akt have been described (3). Previous reports from our laboratory and others demonstrated that one downstream effect of Akt activation is NF-κB-dependent transcription (4), but it is still not known what role NF-κB plays in Akt-dependent biological processes overall. Most previous studies, including our own, relied on gain of function approaches to show that Akt can be sufficient to up-regulate NF-κB-dependent transcription. Thus, the degree to which Akt is actually necessary for NF-κB induction during T cell activation is not clear and, indeed, controversial.

NF-κB family transcription factors reside in the cytoplasm of unstimulated cells as homo- or heterodimers in complexes with the IκB proteins. Upon stimulation, phosphorylation by IKK[2] triggers IκB degradation, which promotes NF-κB factor translocation to the nucleus followed by their binding to and activation of transcription from numerous genes (5). However, the regulation of gene transcription by NF-κB is more complex than this simple model suggests, and it is now clear that multiple levels of regulation exist to yield the known stimulation- and cell type-specific patterns of NF-κB gene expression (6). For example, differences in the magnitude, duration, and/or periodicity of NF-κB activation have been shown to contribute to the up-regulation of different subsets of NF-κB-dependent genes by different receptors (7–11).

Recently, the development of more potent and selective small molecule inhibitors of Akt has facilitated the study of Akt function both *in vitro* and *in vivo*, although these have mostly been explored as possible cancer therapies (12, 13). Here we have employed the compound Akt*i* 1/2, an allosteric inhibitor that stabilizes the inactive conformation of both Akt1 and Akt2 but not Akt3 (14). We previously showed that this compound is an effective inhibitor of Akt activation in T cells, at least under conditions of short-term stimulation (4). We also used the complementary approach of knocking down Akt1 and Akt2 with siRNA. In this study, using targeted gene array analysis, we analyzed the program of NF-κB-dependent gene expression induced during T cell activation and established a subset of genes that requires Akt for its up-regulation. Our findings demonstrate that Akt fine-tunes NF-κB-dependent transcription by the TCR and CD28, with only a subset of genes sensitive to the loss of Akt activity. Importantly, we provide mechanistic evidence that the difference between Akt-sensitive and Akt-insensitive NF-κB target genes is due to quantitative effects of Akt on

* This work was supported, in whole or in part, by NIGMS National Institutes of Health Grant GM030398 (to L. P. K.).
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.
[1] To whom correspondence should be addressed: Dept. of Immunology/BST E-1056, University of Pittsburgh, Pittsburgh, PA 15261. E-mail: lkane@pitt.edu.

[2] The abbreviations used are: IKK, IκB kinase; Ub, ubiquitination; TCR, T cell receptor for antigen.

NF-κB induction. Finally, using this approach, we have identified and validated the proinflammatory cytokine TNF-α as a particularly sensitive target for Akt inhibition in T cells.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-human CD3ϵ and CD28 were from BioLegend (San Diego, CA). Biotin-anti-mCD28 (37.51) and biotin-anti-mCD3ϵ (145–2C11) were from BD Biosciences. Streptavidin and pAkt (S473) antibodies were from Invitrogen. Anti-β-actin and ionomycin were from Sigma. Akt*i* 1/2 and phorbol 12-myristate 13-acetate were from EMD Biosciences (San Diego, CA). Akt siRNA oligos were from New England Biolabs. Recombinant hIL-2 was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (catalog no. 136) from Hoffman-La Roche. Anti-p65 (sc-109x) and anti-IKKγ (sc-8032) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-IκBα and phospho-IKKα/β were from Cell Signaling Technology, Inc. (Danvers, MA). Carma1 antibody was from GenWay (San Diego, CA). Antibodies to Bcl10 and p65 were from Santa Cruz Biotechnology, Inc. Antibody to Lys-63-linked Ub was from Enzo Life Sciences (Farmingdale, NY).

T Cell Lines and Transfections—The D10 T cell clone was maintained in RPMI with 10% heat-inactivated bovine growth serum (HyClone/Thermo Scientific, Waltham, MA), and 25 IU/ml recombinant human IL-2. $CD4^+$ T cells were isolated from lymph nodes and spleens from 6- to 12-week-old DO11.10 TCR transgenic mice with the murine T cell purification kit from Miltenyi Biotec (and stimulated in 24-well plates coated with anti-Syrian hamster IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), anti-mouse CD3 (1 μg/ml, Invitrogen), and anti-mouse CD28 (5 μg/ml, Invitrogen). Th1 cells were differentiated from these cells in the presence of mouse IL-12 (5 ng/ml, BD Biosciences), anti-mouse IL-4 (10 ng/ml; BioLegend), and rhIL2 (25 IU/ml).

For transfection, T cells were resuspended at 35×10^6 cells/ml in RPMI 1640 without supplements. Cells (0.4 ml of cells in a 0.4-cm cuvette) were electroporated in a Gene-Pulser (Bio-Rad) at 250 V and 960 μF and then cultured overnight in 10 ml of complete D10 medium, including IL-2. The next day, live cells were isolated on Lympholyte (Cedarlane Laboratories; Burlington, NC) and recultured for 3–4 h in complete D10 medium, excluding IL-2, before stimulation.

Luciferase Assays—Jurkat T cells were stimulated for 6 h with anti-TCR antibody C305. Luciferase assays were then performed as described previously (4).

Site-directed Mutagenesis—The previously described W80A mutation (15) was made in a murine Akt1 cDNA clone using the QuikChange system from Stratagene (San Diego, CA). The resulting construct was verified by automated sequencing.

Cell Lysis, SDS-PAGE, and Western Blotting—Cell lysates were prepared in lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 1 mM EDTA in addition to protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, aprotinin, and leupeptin, all used according to the manufacturer's recommendations (EMD Biosciences), and phosphatase inhibitors (sodium orthovanadate (EMD Biosciences) and β-glycerol phosphate (Sigma)). For analysis of Ub, lysis buffer was also supplemented with 5 mM *N*-ethylmaleimide. Western blotting was carried out as described previously (4). Western blot analyses were developed with SuperSignal West Pico substrate (Pierce/Thermo Scientific) and imaged on a Kodak Image Station 2000R.

Gene Array Expression Analysis—T cells were left untreated or pretreated with 20 μM Akt*i*1/2 for 1 h and then stimulated with biotinylated anti-CD3/CD28 and streptavidin for 0, 2, 6, and 12 h. Alternatively, T cells were transfected by electroporation with 100 nM Akt Shortcut® siRNA oligos (New England Biolabs). 24 h after transfection, cells were stimulated as above. mRNA was isolated with the ArrayGrade mRNA purification kit (SA Biosciences, Frederick, MD). Using the TrueLabeling-AMP linear RNA amplification kit (SA Biosciences), mRNA was reverse-transcribed to obtain cDNA and converted into biotin-labeled cRNA using biotin-16-UTP (Roche) by *in vitro* transcription. Prior to hybridization, cRNA probes were purified with the ArrayGrade cRNA clean-up kit (SA Biosciences) and then hybridized to pretreated Mouse NF-κB Signaling Pathway Microarray OMM-025 (SA Biosciences). Following washing, cRNA binding was detected using alkaline phosphatase-conjugated streptavidin and ECL substrate. The signal was detected with a Kodak Image Station 2000R. The image data were transformed into numerical data and analyzed using the GEArray Expression Analysis Suite (SA Biosciences).

Real-time RT-PCR—Total RNA was reverse transcribed using the RT^2 first strand kit (C-03, SA Biosciences). 18 S rRNA was chosen as the reference gene for normalization. Real-time PCR was performed with a StepOnePlus real-time PCR system (Applied Biosystems, Inc., Foster City, CA) using RT^2 SYBR Green/ROX quantitative PCR master mixes (SA Biosciences). PCR primers were from SA Biosciences. PCR products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no byproducts were formed.

Multiplex Bead-based and ELISA Measurement of Cytokines—Secreted levels of seven cytokines in the supernatants of stimulated cells were measured with a custom kit from Bio-Rad using the Luminex platform. Alternatively, the level of secreted TNF-α in D10 T cell or bone marrow-derived macrophage cell culture supernatants in some experiments was measured using a commercial mouse TNF-α ELISA kit from BioLegend. Whole heparinized human blood was obtained from the Central Blood Bank (Pittsburgh, PA). Anti-CD3ϵ (1 μg/ml), anti-CD28 (10 ng/ml), and IL-2 (1:2000 dilution of recombinant human IL-2) were added to whole blood diluted 1:1 in RPMI 1640 and incubated at 37 °C for 18 h.

Microwell NF-κB DNA Binding Assay—Binding of nuclear p65/RelA to an NF-κB DNA consensus sequence was measured using a commercially available kit (NF-κB p65 EZ-TFA transcription factor assay (Millipore, Temecula, CA). This method was used as an alternative to the electrophoretic mobility shift assay and has been reported to be sensitive and specific (16).

Chromatin Immunoprecipitation—ChIP assays for NF-κB p65 were performed using a ChIP assay kit (USB, Cleveland, OH) according to the manufacturer's recommendations. PCR was performed using primers complementary to two sites

flanking an approximately 300-bp fragment of the murine TNF-α promoter between nucleotides −434 and −722 relative to the transcription start site and encoding κB sites 2 and 2a: 5′-AGTCATACGGATTGGGAGAAATCCTG-3′ (forward) and 5′AGTTCTTGGAGGAAGTGGCTG-AAGGCA-3′ (reverse). For IκBa, the primers were ACTCTTGGGTTTTCAA-AAAGATCA (forward) and GCCAGTCAGACTAGAAAAA-GAACTG (reverse). The products were analyzed on a 2% agarose gel.

IKK Kinase Assay—IKK kinase assays were performed essentially as described previously (4, 17). Briefly, the IKK complex was immunoprecipitated from unstimulated or stimulated D10 T cells with an antibody to IKKβ. Immunoprecipitants were then washed into kinase buffer with GST-IκB as a substrate. Reactions were separated on 10% SDS-PAGE gels and imaged by autoradiography.

Collagen-induced Arthritis—Collagen-induced arthritis (CIA) was induced as described previously (18) in accordance with University of Pittsburgh Institutional Animal Care and Use Committee procedures.

Statistical Tests—Results are presented as the mean ± S.D. Where indicated, *p* values were calculated using a Student's two-tailed *t* test, on the basis of at least three replicate experiments.

RESULTS

We first confirmed which isoforms of Akt are expressed in T cells. Thus, we found that T cells express both Akt1 and Akt2 but do not express detectable levels of Akt3 (Fig. 1*A*). We next determined whether the inhibitory activity of Akt*i* 1/2 is maintained over the time course of T cell stimulation relevant for the experiments performed below. Treatment of a murine T cell clone with Akt*i* 1/2 (10 μM) resulted in long-lasting inhibition of Akt activation (at least 12 h), as read out by phosphorylation of Ser-473 (Fig. 1*B*). To help confirm the specificity of the Akt inhibitor, we tested its efficacy in an NF-κB reporter assay using cells transfected with WT Akt or a form of the kinase described previously to be resistant to the effects of Akt*i* 1/2 (15). Thus, consistent with our previous findings (4), Akt*i* 1/2 partially inhibited activation of an NF-κB reporter by TCR stimulation (Fig. 1*C*). This effect was also pronounced in T cells cotransfected with WT Akt but not Akt mutated so as to be resistant to the effects of the inhibitor (W80A). Although this and other findings point to Akt*i* 1/2 being a very specific inhibitor of two of the three mammalian Akt isoforms, Akt1 and Akt2 (14, 19), we were concerned about possible off-target effects. Therefore, we also employed siRNA to knock down the expression of Akt1 and Akt2 in T cells. As shown in Fig. 1*D*, transfection of T cells with oligos specific for Akt1 and Akt2 resulted in significant knockdown of Akt protein, peaking at about 24 h.

To test the hypothesis that Akt is selectively involved in the up-regulation of a subset of NF-κB-dependent genes in T cells, we employed targeted microarrays ("NF-κB Signaling Pathway Oligo Array" under "Experimental Procedures") specific for 113 genes that are known targets and regulators of NF-κB signaling. Thus, T cells were pretreated with 10 μM Akt*i* 1/2 for 1 h or transfected with Akt-specific siRNA (for 24 h) and then stimulated with anti-CD3/CD28 antibodies followed by RNA isolation and preparation for array analysis. Stimulation of D10 T cells led to the up-regulation of a number of genes represented on the arrays (Fig. 2 and supplemental Figs. S1–S4). After pretreatment with Akt*i* 1/2 or transfection of Akt1/2-specific siRNA oligos, only a subset of these genes was impaired in its up-regulation (Fig. 2 and supplemental Figs. S1–S4). Notable among these were the genes encoding the cytokines TNF-α, LIGHT (*Tnfsf14*), IL-6, IL-10, and GM-CSF (*Csf2*), which were similarly affected by both the Akt inhibitor and siRNA treatment (genes highlighted in *bold type*). Importantly, some known NF-κB targets were not affected by either the Akt inhibitor or siRNA, for example, the genes encoding IκBa (*Nfkbia*)

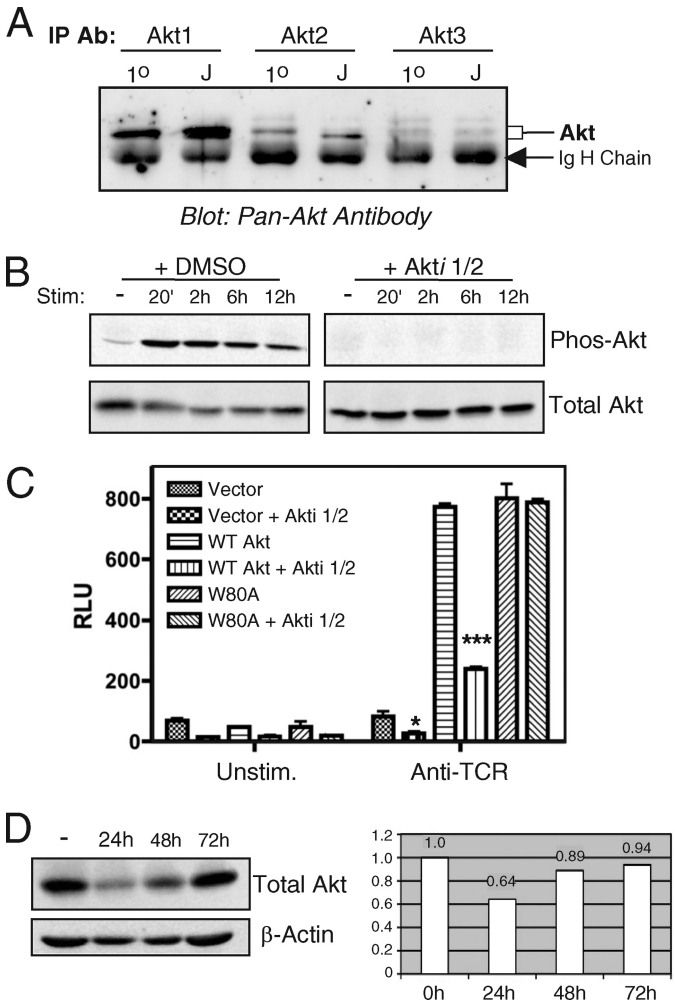

FIGURE 1. **Validation of Akt inhibition strategies.** *A*, expression of Akt isoforms in T cells. Primary murine T cells (*1°*) or Jurkat T cells (*J*) were lysed and immunoprecipitated (*IP*) with the indicated Akt isoform-specific antibodies (*Ab*) and then analyzed by Western blotting with a pan-Akt antibody. *B*, D10 T cells were stimulated (*Stim*) with anti-CD3/CD28 antibodies for the indicated times in the absence (*left panels*) or presence (*right panels*) of Akt*i* 1/2. Cell lysates were analyzed by SDS-PAGE and Western blotting for phospho-Akt (Ser-473) (*upper panels*) followed by total Akt (*lower panels*). *DMSO*, dimethyl sulfoxide. *C*, specificity of Akti 1/2 effect on NF-κB reporter demonstrated with an inhibitor-resistant Akt allele. Jurkat T cells were transfected with an NF-κB luciferase reporter and the indicated constructs. The next day, cells were stimulated with anti-TCR antibody followed by determination of luciferase activity. *RLU*, relative luciferase units. *, $p < 0.05$; ***, $p < 0.001$. *D*, D10 T cells were transfected with siRNA oligos specific for murine Akt1 and Akt2. Lysates were made at the indicated times after transfection and analyzed by SDS-PAGE for total Akt expression (*upper panel*) or β-actin as a loading control (*lower panel*). Results of the Akt blot are quantified in the *right panel*.

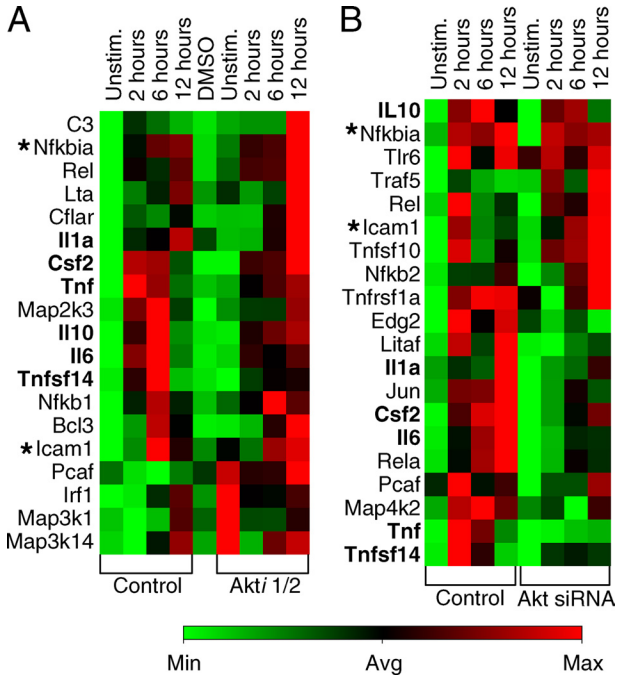

FIGURE 2. **Selective effects of Akt inhibition or knock-down on NF-κB signature genes.** Representative partial heat maps from D10 T cells were stimulated with anti-CD3/CD28 antibodies and RNA was extracted, followed by analysis of expression of NF-κB pathway genes as described under "Experimental Procedures." *A*, partial heat map of gene expression changes with or without treatment with Akt*i* 1/2. *B*, partial heat map of gene expression changes after transfection with control or Akt1/2-specific siRNA. *, genes not affected by Akt inhibition or knock-down.

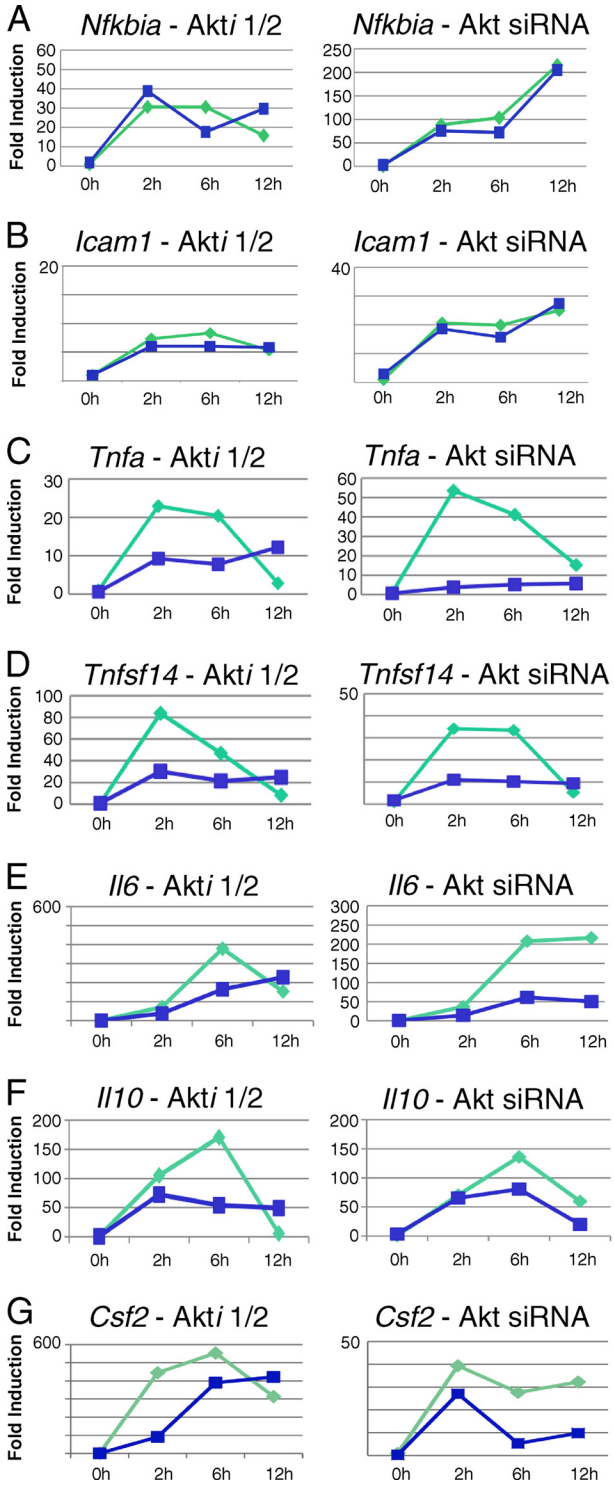

FIGURE 3. **Quantitative RT-PCR analysis of Akt-dependent genes in the NF-κB pathway.** D10 T cells were stimulated with anti-CD3/CD28 antibodies, and RNA was extracted, followed by quantitative RT-PCR analysis of the effects of Akt*i* 1/2 (*left panels*) and Akt siRNA (*right panels*) on selected NF-κB pathway genes. *A*, IκBa. *B*, ICAM1. *C*, TNF-α. *D*, TNFSF14 (LIGHT). *E*, IL-6. *F*, IL-10. *G*, CSF2 (GM-CSF). Results are representative of three separate experiments for each panel. *Green diamonds*, solvent or control siRNA; *blue squares*, Akt*i* 1/2 or Akt1/2 siRNA treatment.

and ICAM1 (Fig. 2 (genes highlighted with *asterisks*) and supplemental Figs. S1 and S3).

To validate the results obtained with the microarrays, we examined the expression of the genes highlighted above with quantitative RT-PCR. The quantitative RT-PCR analysis confirmed our initial observation that increases in the messages encoding IκBα and ICAM1 were not affected by Akt inhibition or knockdown (Fig. 3, *A* and *B*). Importantly, these experiments also confirmed that the CD3/CD28-induced expression of the genes encoding TNF-α (Fig. 3*C*), LIGHT (*D*), IL-6 (*E*), IL-10 (*F*) and GM-CSF (*G*) were all inhibited by treatment with 10 μM Akt*i* 1/2 (*left panels*) or transfection with Akt1/2 siRNA oligos (*right panels*). Thus, modulation of Akt activity or expression indeed results in selective effects on known NF-κB target genes. Given the particularly striking difference between the effects of Akt modulation on up-regulation of the genes encoding IκBα *versus* TNF-α, we examined these genes more carefully for a specific connection to their transcriptional regulation by NF-κB.

NF-κB is a well known regulator of TNF-α transcription through multiple NF-κB-binding sites in the TNF-α promoter (20, 21). We performed a ChIP analysis of the endogenous TNF-α promoter before and after CD3/CD28 stimulation, with or without the inhibitor Akt*i* 1/2. As shown in Fig. 4*A* (*left panel*), stimulation of D10 T cells led to increased NF-κB p65 binding to the TNF-α promoter. This activity was significantly impaired in the presence of Akt*i* 1/2, with the effects over three experiments quantified in Fig. 4*A* (*right panel*). To further solidify the link between Akt, NF-κB, and gene expression, we performed a ChIP analysis for p65 binding to the promoter of the IκB gene (*Nfkbia*), expression of which was not affected by Akt inhibition (Figs. 2 and 3). Thus, as shown in Fig. 4*B*, CD3/

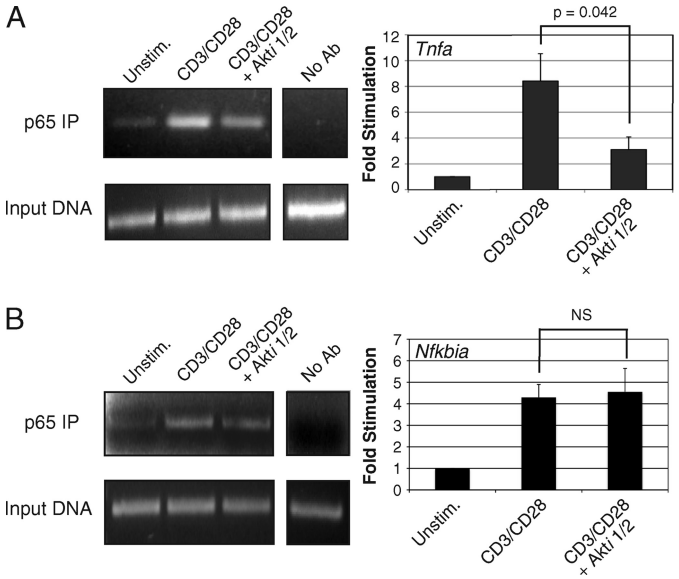

FIGURE 4. **Effects of Akt inhibition at the level of NF-κB promoter binding.** *A* and *B*, chromatin immunoprecipitation was performed on the promoters of the genes encoding TNF-α (*A*) or IκBα (*B*), as described under "Experimental Procedures," after stimulation of D10 T cells for 6 h in the absence or presence of Akt*i* 1/2 (10 μM). *Left panels*, representative ChIP experiments of three that were performed for each gene. *Right panels*, quantitation of ChIP results over three experiments, represented as the average fold stimulation ± S.D. compared with unstimulated cells.

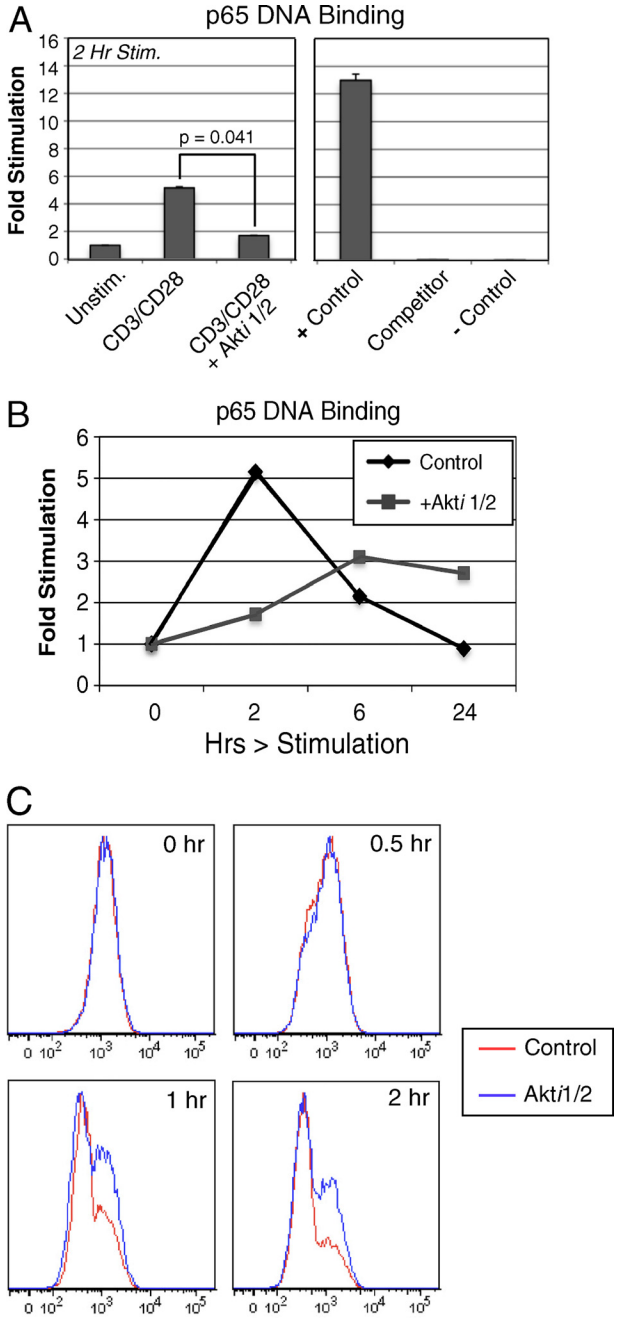

FIGURE 5. **Akt inhibition alters the kinetics of NF-κB activation downstream of CD3/CD28.** *A*, D10 T cells were stimulated (*Stim*) for 2 h as indicated. Nuclear extracts were prepared and analyzed for p65 binding to an NF-κB consensus oligonucleotide (*left panel*). The *right panel* contains controls, including a positive control extract, stimulated cell extract analyzed in the presence of a non-labeled competitor oligonucleotide, and a negative control (no probe). Results shown are the average fold stimulation ± S.D. of three independent experiments. *B*, time course of NF-κB p65 nuclear entry with or without Akt*i* 1/2 treatment. *C*, effects of Akt inhibition on CD3/CD28-induced IκBα degradation. D10 T cells were stimulated with anti-CD3/CD28 antibodies without or with 10 μM Akt*i* 1/2. Cells were fixed, permeabilized, and stained for intracellular IκB and flow cytometry.

CD28 stimulation led to increased p65 binding to the IκB promoter, which was not inhibited by Akt*i* 1/2. These results demonstrate that inhibition of Akt1 and Akt2 inhibits transcription from the TNF-α promoter in T cells, at least in part through decreased NF-κB DNA binding. However, binding of p65 to the IκB promoter under the same conditions was not affected. Thus, the transcriptional differences described above were due at least in part to direct effects on NF-κB promoter binding.

To better understand the selective effects of Akt inhibition/knockdown on NF-κB promoter binding, we further probed the role of Akt in the NF-κB signaling pathway. As shown in Fig. 5*A*, Akt*i* 1/2 significantly (but not completely) inhibited nuclear entry and DNA binding of p65 induced by stimulation with anti-CD3/CD28 antibodies, at least at this 2-h time point. However, when the kinetics of the response were examined, we did observe an eventual increase in p65 nuclear entry and DNA oligo binding at later time points in the presence of the Akt inhibitor (Fig. 5*B*), despite the fact that inhibition of Akt activity was maintained (Fig. 1*B*). Consistent with the altered kinetics of p65 nuclear entry and DNA binding, we found that inhibition of Akt led to a delay, but not absence, of CD3/CD28-induced degradation of IκBα (Fig. 5*C*). Thus, consistent with previous studies on the selective control of gene expression by different NF-κB-activating receptors (6), Akt-mediated differences in NF-κB kinetics appear to be translated into specific effects on CD3/CD28-mediated gene expression.

We next worked backwards in the NF-κB pathway to better understand where Akt inhibition was impinging on normal NF-κB induction. IκBα degradation requires phosphorylation by the IKK complex (5). Indeed, when we measured IKK kinase activity with an *in vitro* assay, we noted that CD3/CD28-induced IKK activity was impaired in the presence of Akt*i* 1/2 (Fig. 6*A*). The dynamic range of this kinase assay is not particularly large in our experience, which may account for the apparently larger effect on kinase activity compared with IκB degradation. Activation of the IKK complex requires both phosphorylation of IKKα/β and Ub of IKKγ/NF-κB essential modulator. Blotting stimulated T cell lysates with a phospho-

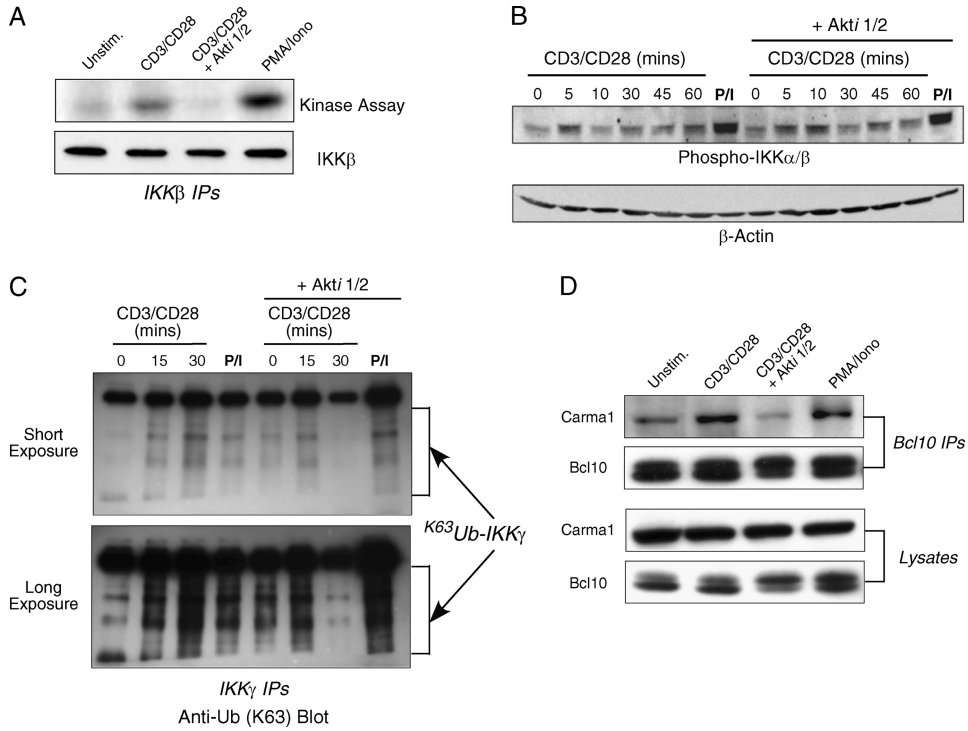

FIGURE 6. **Akt inhibition alters signaling to NF-κB from CD3/CD28.** *A*, lysates were made and immunoprecipitated (*IP*) for the IKK kinase complex using an antibody to IKKγ/NEMO. Kinase assays were performed and run out on SDS-PAGE gels, transferred to PVDF, and exposed to x-ray film (*top panel*). Blots were then probed for immunoprecipitated IKKβ (*bottom panel*). *B*, cell extracts were analyzed by Western blotting for phospho-IKKα/β (*top panel*) with β-actin as a loading control (*lower panel*). *C*, stimulated D10 cells were lysed and subjected to *IP* with anti-IKKγ/NEMO antibody. Immunoprecipitates were washed with radioimmune precipitation assay buffer followed by Western blotting with an antibody to Lys-63-linked ubiquitin. *D*, cells were stimulated as indicated, and Bcl10 immunoprecipitates were blotted for coimmunoprecipitated Carma1 (*first panel*) or for Bcl10 (*second panel*), as were whole cell lysates (*third* and *fourth panels*). Results in each part are representative of at least three experiments.

specific antibody to IKKα/β revealed little if any difference in magnitude or kinetics of phosphorylation in the presence or absence of the Akt inhibitor (Fig. 6*B*). However, when we examined Lys-63-linked Ub of IKKγ, we did note effects of the inhibitor. As shown in Fig. 6*C*, anti-CD3/CD28 or phorbol 12-myristate 13-acetate /ionomycin stimulation increased the amount of Lys-63-Ub on IKKγ. Use of Akt*i* 1/2 in this assay revealed two differences: a slight but reproducible increase in the basal amount of Ub and a decrease in the induced Ub, particularly at the 30-min time point. Because CD3/CD28-mediated Ub of IKKγ requires assembly of a multiprotein complex containing Carma1and Bcl10 (22), we reasoned that formation of this complex might be impaired by Akt inhibition. T cells were stimulated with or without Akt*i* 1/2, and endogenous Bcl10 was immunoprecipitated, followed by Western blotting for Carma1. Thus, as shown in Fig. 5*E*, stimulation with anti-CD3/CD28 antibodies led to an increase in the amount of Carma1 coimmunoprecipitated with Bcl10, and this was severely impaired in the presence of Akt*i* 1/2. As with the IKK kinase assay discussed above, the dynamic range of this coimmunoprecipitation assay is rather modest, which likely explains the apparent severity of the inhibition.

The results discussed above suggest that Akt inhibition may have selective effects on cytokine production by effector T cells. To address this further at the protein level, we determined the role of Akt in the secretion of a panel of cytokines known to be made by the D10 T cell clone using a multiplex bead-based approach. Thus, we stimulated D10 T cells with anti-CD3/CD28 antibodies in the presence of different concentrations of Akt*i* 1/2 (1, 10, or 20 μM) to determine the relative sensitivity of different cytokines to the inhibitor. As shown in Fig. 7*A*, there was a preferential effect on certain cytokines at lower doses of the compound. Interestingly, some of the canonical Th2 cytokines, including IL-4, IL-5, and GM-CSF but not IL-13, appeared to be more resistant to the effects of Akt*i* 1/2 relative to IL-6 and especially TNF-α. A comparative analysis where the data are normalized (Fig. 7*B*) suggests that these cytokine break down into two classes with respect to sensitivity to Akt inhibition, resulting in a shift of the dose response curve of approximately one log.

Given the clinical relevance of TNF-α (23), and the complex post-transcriptional control of TNF-α message (24, 25), we further assessed the effects of Akt inhibition on TNF-α protein in T cells from different sources. To confirm the results obtained with the multiplex approach in Fig. 7, we measured TNF-α secretion with a sensitive ELISA. As shown in Fig. 8*A*, TNF-α secretion by D10 T cells was profoundly inhibited by Akt*i* 1/2, consistent with results discussed above. In addition, we used this approach to measure TNF-α production by T cells transfected with siRNA against Akt1 and Akt2. Consistent with the less-than-complete knockdown of Akt (Fig. 1), we observed significant, although not complete, inhibition of TNF-α production. Similar results were observed with polyclonal Th1 T cells, although the overall level of TNF-α production by these cells was lower (Fig. 8*B*).

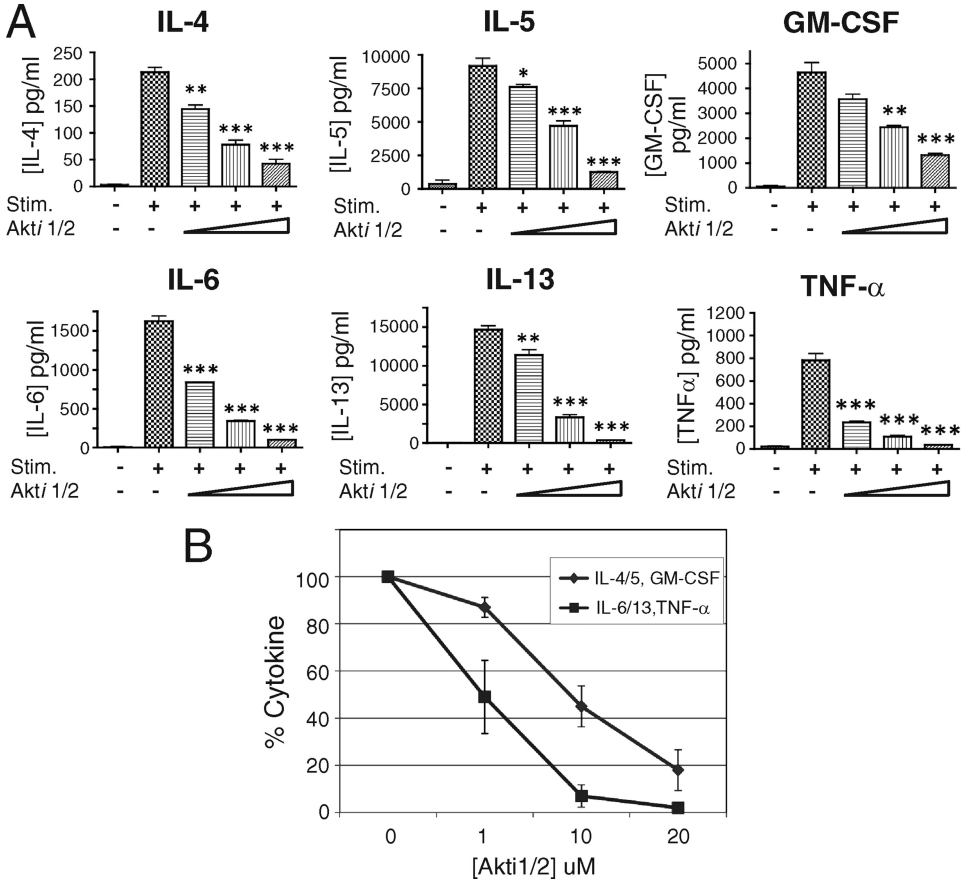

FIGURE 7. **Differential effects of Akt inhibition on T cell cytokine production.** *A*, D10 T cells were stimulated (*Stim*) for 24 h with anti-CD3/CD28 monoclonal antibodies in the absence or presence of increasing concentrations of Akt*i* 1/2 (1, 10, and 20 mM). Supernatants were analyzed for the indicated cytokines with a bead-based multiplex system. Results are the average ± S.D. of triplicate wells and are representative of three experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, results for the indicated cytokines were normalized to the starting time point and graphed together.

Next, we examined the effects of Akt inhibition on production of TNF-α following anti-CD3/CD28 stimulation of whole human blood. Such an assay is often used for identifying the potential clinical utility of small molecule inhibitors of kinases, in the more complex environment of whole blood (26). As shown in Fig. 8*C*, anti-CD3/CD28 stimulation of whole blood resulted in production of TNF-α, presumably mostly from T cells, and this was significantly inhibited by Akt*i* 1/2 (*white bars*). Although there was substantially more TNF-α produced after stimulation of whole blood cultures with phorbol 12-myristate 13-acetate and ionomycin, which bypass receptor-proximal signaling pathways, this was not inhibited by Akt*i* 1/2 (Fig. 8*C*, *black bars*). This important control helps to confirm the specificity of the Akt inhibitor and its lack of general toxicity. Finally, we examined the effect of Akt inhibition on antigen-specific TNF-α production by T cells from a mouse model of rheumatoid arthritis. Thus, mice were immunized with type II collagen and followed for development of joint swelling (18, 27). Upon identification of animals with inflammation, draining lymph nodes and spleens were harvested, and cells were restimulated *in vitro* with antigen. As shown in Fig. 8*D*, addition of antigen (type II collagen) to lymph node cultures (*white bars*) led to increased secretion of TNF-α, compared with unstimulated (no antigen) cultures. This secretion was inhibited by all doses of Akt*i* 1/2 (from 1–20 μM, *shaded bars*). We also noted significant inhibition by Akt*i* 1/2 of anti-CD3/CD28-stimulated TNF-α production in these cultures of murine lymph node cells (Fig. 8*D*). Because a recent publication suggested that Akt*i* 1/2 might act in part through inhibition of calcium/calmodulin dependent protein kinase (28), we also stimulated T cells in the presence of the potent CaMK inhibitor KN-93. However, this compound had no effect on TNF-α production (data not shown), providing additional evidence that Akt*i* 1/2 blocks TNF-α production as a result of inhibition of Akt activity.

DISCUSSION

In this study, we set out to clarify the role of the serine/threonine kinase Akt in CD3/CD28-induced NF-κB activity. Our data are consistent with a model whereby Akt is not a canonical member of the pathway leading from CD3/CD28 to NF-κB, but rather this kinase appears to modulate flux through the pathway. Importantly, these conclusions were based not only on the use of a selective small molecule inhibitor of Akt but also siRNA-mediated knockdown of Akt1 and Akt2. Although earlier gain of function studies suggested a role for Akt in regulating the NF-κB pathway during T cell activation (4, 17, 29–31), the extent and mechanism of Akt involvement in NF-κB activation have not been clear. Our data show for the first time that Akt activity is required for induction of a subset

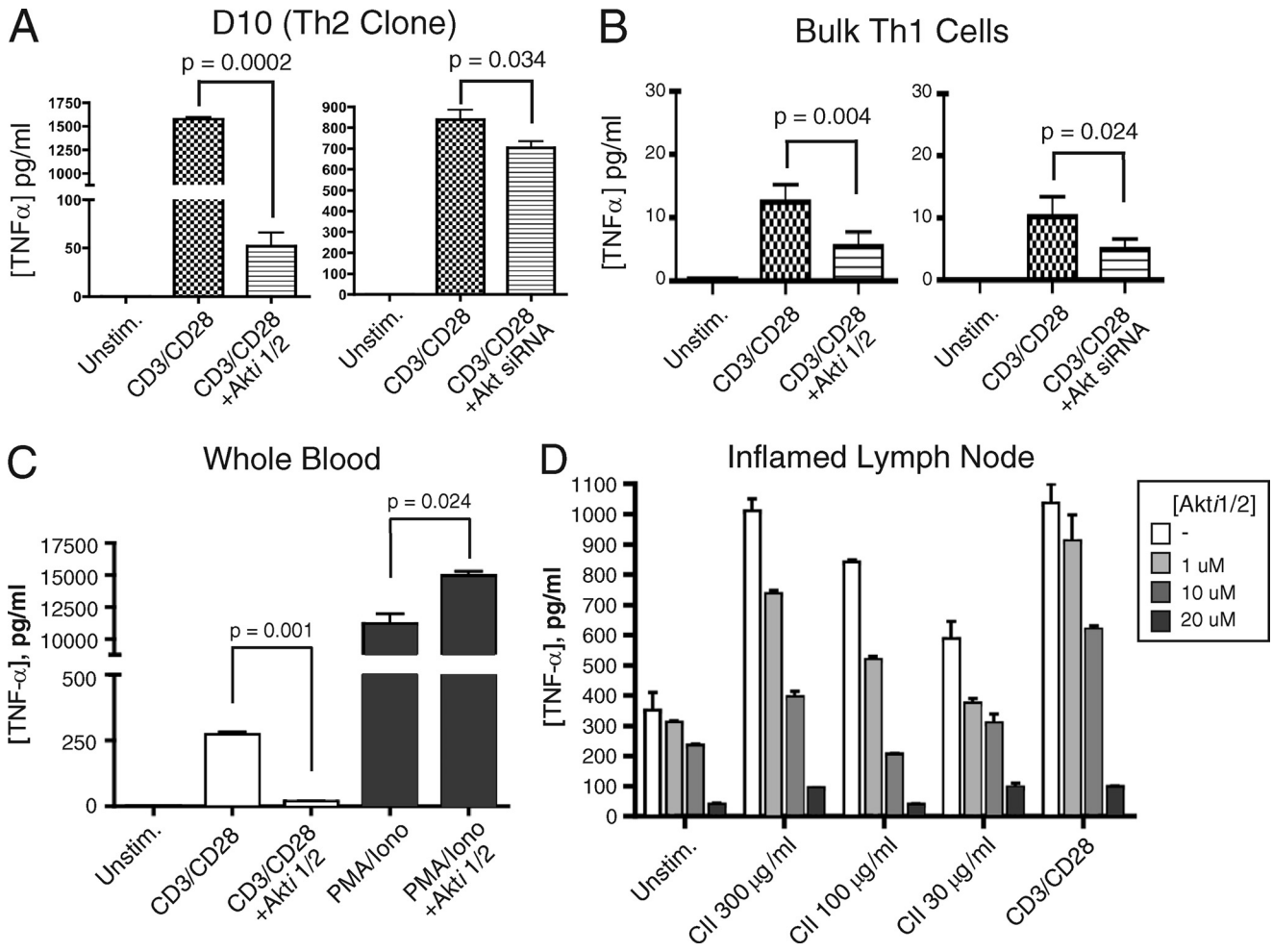

FIGURE 8. **Requirement for Akt in TNF-α production by T cells from multiple sources.** *A* and *B*, comparison of Akt*i* 1/2 and Akt siRNA effects on TNF-α production. The murine Th2 cell clone D10 (*A*) or bulk murine Th1 cells (*B*) were stimulated as indicated, following 30-min pretreatment with Akt*i* 1/2 or 24-h transfection with Akt1/2 siRNA oligos. After 24-h stimulation, murine TNF-α was measured in cell-free supernatants by ELISA. *C*, whole human blood was stimulated with anti-CD3/CD28 antibodies for 18 h, followed by analysis of hTNF-α concentration by ELISA. Results shown are the average ± S.D. of triplicate wells from a single experiment, representative of four experiments performed with two different donors. *D*, lymph node cells from mice immunized with type II collagen (*CII*) were restimulated *in vitro* without (*white bars*) or with (*shaded bars*) Akt*i*1/2 in increasing concentrations (1, 10, and 20 μM). The culture supernatant was assayed for TNF-α by ELISA. Results are the average of triplicate points from a single experiment, representative of three that were performed.

of NF-κB-dependent genes during T cell activation, at least in part through effects on p65/RelA nuclear entry and DNA binding. One of the most consistently and potently inhibited genes was that encoding TNF-α, a particularly relevant target for immune modulation.

Our current model, on the basis of this and previous work (4), is that Akt exerts a quantitative effect upstream of IKK activation but downstream of CD3 and CD28, resulting in an amplification of the NF-κB response (Fig. 9). Thus, knockdown or small molecule inhibition of Akt dampens but does not completely impair NF-κB activity, as read out by IκBα degradation and p65 nuclear entry. In particular, the assessment of p65 nuclear entry and DNA binding ability (at least to an oligo) revealed a shift in the kinetics of NF-κB activity in the absence of Akt activation. Several groups have elegantly demonstrated that quantitative effects on NF-κB activity can selectively impact the expression of certain NF-κB target genes (7, 8, 11, 32, 33) and that this might underlie the observation that different NF-κB-activating stimuli up-regulate the expression of distinct subsets of genes. However, this possibility has not been addressed previously with antigen receptor signaling in T or B cells, nor has it previously been ascribed to a single intermediate in a signaling pathway, as we have demonstrated here with Akt.

Working upstream from p65 and IκB, we show that IKK kinase activity is inhibited by Akt*i* 1/2, as are IKKγ/NEMO ubiquitination and Carma/Bcl10/MALT1 complex assembly. However, IKKα/β phosphorylation appears to be unaffected by Akt inhibition. Thus, on the basis of previous work of Lin and colleagues (22), our data point to a defect at or upstream of the CBM complex when Akt is inhibited. This conclusion is also consistent with a previous study from our group that demonstrated a requirement for Carma1 expression in mediating the activation of NF-κB by ectopic Akt expression (4). Although these findings point to possible phosphorylation of one or more CBM components by Akt, we have not yet succeeded in identifying a direct target for Akt phosphorylation in the CBM complex. Carma1 is one possibility because it is known to be regulated by multiple phosphorylation events (34), but Carma1 contains no obvious sites of Akt phosphorylation. This is an important question for future studies to address.

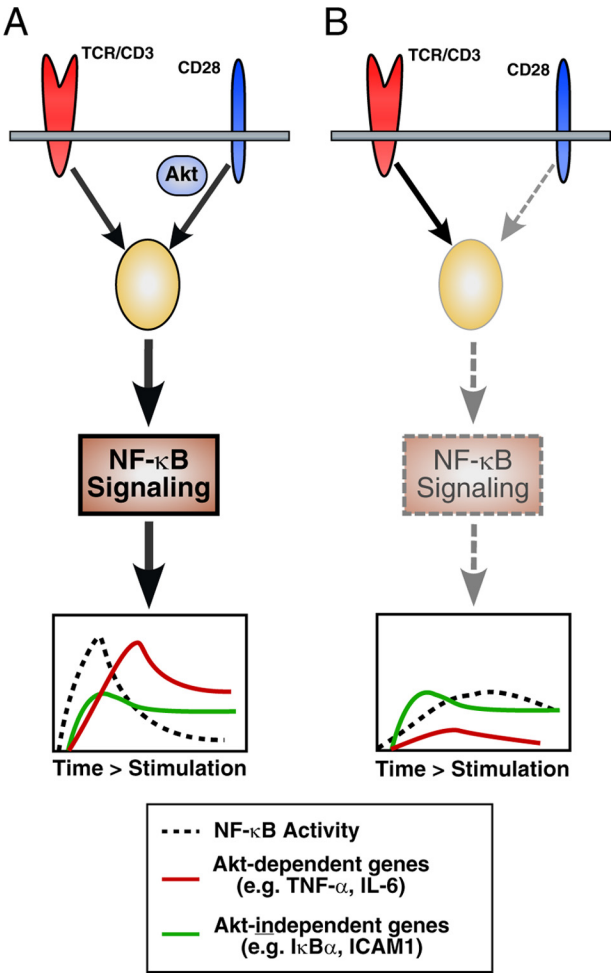

FIGURE 9. **Model for the role of Akt in regulating NF-κB-dependent transcription downstream of the TCR and CD28.**

Intriguingly, we noted that up-regulation of the gene encoding IκBα was not significantly affected by Akt inhibition or knockdown, consistent with previous observations in other systems that IκBα is less sensitive to the overall magnitude and/or duration of NF-κB activity (7). Because IκBα is an important feedback regulator, overall weaker stimulation of the pathway (*e.g.* in the presence of the Akt inhibitor), coupled with relatively normal up-regulation of IκBα, may result in a more dramatic effect on those genes that require the most robust NF-κB activity (such as TNF-α). Thus, our findings are also consistent with previous reports that NF-κB-dependent TNF-α up-regulation is relatively sensitive to the levels of NF-κB activity (11). Although T cells are not usually discussed as major producers of TNF-α during autoimmunity, increased production of TNF-α among antigen-specific T cells has been observed in the collagen-induced arthritis model (35). In addition, there are a number of other settings in which T cell production of TNF-α appears to be clinically relevant. Production of TNF-α by bone marrow T cells has been linked to bone loss associated with estrogen deficiency (36, 37). In addition, the presence of TNF-α-producing T cells correlates with disease severity and progression in multiple sclerosis (38, 39). Finally, the production of TNF-α by alloreactive T cells was shown to predict allograft rejection (40).

There continues to be interest in the potential clinical utility of small molecule inhibitors to target inflammation, particularly in the context of high levels of proinflammatory cytokines like TNF-α and IL-6. Although protein drugs that neutralize TNF-α have been successful in the treatment of diseases like rheumatoid arthritis (41), these therapies are expensive and difficult to produce and deliver, and their continued use can lead to the development of neutralizing antibodies that negate their efficacy in some patients. For these reasons, a number of small molecule compounds that show promise in inhibiting either the production or action of proinflammatory cytokines like TNF-α continue to be actively pursued (42, 43). Although inhibition of Akt in various settings has so far been disappointing because of deleterious effects on metabolic pathways, our studies provide proof of principle that partial inhibition of the NF-κB pathway may allow for more selective modulation of downstream gene expression.

Acknowledgments—We thank P. Changelian for advice on the whole blood stimulation assay and S. Gaffen for critical reading of the manuscript.

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